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Purification and Some Properties of Chitinase from *Aspergillus carneus*

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ABSTRACT

An extracellular chitinase from *Aspergillus cerneus* was purified by ammonium sulphate precipitation, gel filtration through Sephadex G-100, preparative HPLC chromatography and large slabs of polyacrylamide gel electrophoresis.The mol wt of the enzyme was estimated to be 25000 by SDS gel electrophoresis, and it contained 9.37% (w/w) carbohydrate residue, as glucose. The pattern of its amino acid composition showed high contents of asparagine, serine, and threonine. The enzyme was active at pH 5.2 and 50°C. The K_m value of the enzyme was 4.37 m*M* (expressed as *N*-acetylglucosamine). The enzyme was stable at pH 3–9, whereas it was unstable at 70°C or more. Calcium and Mg ions slightly activated the enzyme, whereas Hg^{2+}, I_2, and *p*-chloromercuribenzoate inhibited the enzyme activity. The enzyme hydrolyzed chitin, colloidal chitin, glycol chitin, and chitooligsaccharides, but did not hydrolyze chitosan, starch, xylan, inulin, and cellulose. The lysis of *A. niger* and *Micorcoccus lysodeikticus* cell walls by the action of the enzyme was also investigated.

Index Entries: Chitinase; purification; properties; *Aspergillus carneus*.

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INTRODUCTION

Chitin is a polymer of *N*-acetylglucosamine. It is found in nature as structural polysaccharide in fungal cell walls, the exoskeleton of arthropods, outer shell of crustaceans, and nematodes, and so on (*1,2*). In recent years, much interest was focused on chitin and its application. For instance, this biopolymer was being used successfully in biotechnology, agriculture, and for medical purposes (*2,3*).

Chitin is hydrolyzed to its monomer (*N*-acetylglucosamine) synergistically by multienzyme system. Of these: Endochitinase (EC 3.2.1.14), which hydrolyses chitin randomly to chitooligomers, whereas chitobiase (EC 3.2.1.30) acts on the dimer chitobiose (*4*). The involvement of exochitinase, which hydrolyzes the polymer from the non-reducing end, has also been reported (*5,6*).

Chitinase is produced and isolated in mono component by a variety of microorganisms such as: *Vibro* sp. (*7*): *Saccharomyces cerevisiae* (*8*); *Serratia marcescens* (*9*); *Verticillium albo-atrum* (*10*), and *Streptomyces cinereoruber* (*11*).

We previously isolated a microorganism having chitinase activity, and it was identified as *Aspergillus carneus* (*12*). This strain produced an inducible chitinase system. This paper deals with the purification, characterization, and properties of the chitinase from *A. carneus*.

MATERIALS AND METHODS

Microorganisms

Aspergillus carneus was isolated and identified by The International Commonwealth Mycological Institute, Kew, Surrey, London (*12*), it was maintained in Potato Dextrose Agar (PDA) at 5°C.

Enzyme Production

The culture medium for enzyme production has the following composition (g/L): Colloidal chitin (Sigma), 10.0, yeast extract, 3.0, KH_2PO_4, 1.5, $MgSO_4$, 0.5, and KCl, 0.5. The pH was adjusted to 5.0. For preparation of inoculum 1 mL of 7-d-old culture on PDA (8×10^7 spores/mL) was transferred to 50 mL of the growth medium in 250 mL Erlenmeyer flasks and incubated on a rotary shaker (180 rpm) for 5 d. Cultivation was made in 250 mL Erlenmeyer flasks each containing 50 mL of sterile medium. One mL of the inoculum was transferred to the growth medium. The flasks were incubated at 28°C on a rotary shaker (180 rpm) for 7 d.

Chitinase Activity

One mL of enzyme was incubated with 1.0 mL of 1.0% (w/v) colloidal chitin (Sigma) in 0.05*M* acetate buffer pH 4.5. The reaction mixture was incubated at 45°C for 60 min. The reducing sugar was measured by the method of Reissig et al. (*13*) using standard curve of *N*-acetylglucosamine. One unit of enzyme activity (U) is defined as the amount of enzyme required to produce one μ mole of *N*-acetylglucosamine/min.

Ultrafiltration

Ultrafiltration was carried out by using Millipore Pellicon Cassette System, polysulphone membrane, porosity of MW 5000. Filtration rate was 10 mL/min.

Enzyme Purificatioan

Purification by HPLC

This was done by HPLC (Waters 740 B) using a preparative protein column LKB Glasspak, TSK G 2000 SW (30×600 mm) and UV detector (Waters M-490). The column was equilibrated with 20 m*M* Tris-HCl buffer, pH 8.5, containing 20 m*M* NaCl. The protein was eluted with linear gradient of 10–500 m*M* NaCl in 20 m*M* Tris-HCl buffer, pH 8.5. The flow rate was 5 mL/min.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out in vertical slab-gel prepared by modifying the method of Laemmli (*14*) on 7.5% polyacrylamide (2.5% crosslinkage) at pH 8.5 in nondenaturing system in several runs. After the electrophoresis, the slab gels were cut into two parts, one for staining with Coosmassie Brilliant Blue R-250 to localize the protein bands, and another for the determination of chitinase activity. The active bands were cut into 3 mm slices, and extracted overnight at 4°C with 0.05*M* acetate buffer, pH 5.0.

Determination of Molecular Weight

This was done by the method of Weber and Osborn (*15*) using bovine serum albumin (67000), ovalbumin (47000), pepsin (35000), and lysozome (14000) as standard proteins.

Amino Acid Analysis

About 300 μg of purified protein was hydrolyzed in a sealed tube for 24 h at 110°C with 6*M* HCL. The hydrolyzate was analyzed by Hitachi High Speed Amino Acid Auto Analyzer 835.

Substrate Specificity

One mL of enzyme solution was incubated with 1 mL of each substrate in acetate buffer (0.05*M*, pH 5.2) 45°C for 60 min. The resulting reducing sugars from chitin or chitooligsaccharides were determined by the method of Rondle-Morgan (*16*) (as glucosamine). The sugars obtained from chitosan and other substrates were determined as glucose by the method of Somogyi (*17*). One unit of enzyme activity was defined as the amount of the enzyme which released one μmol of monosaccharides/min.

Chromatography of Hydrolysis Products

The hydrolysis products were detected by descending paper (Whatman no. 3) chromatography in solvent system (*n*-bu anol/acetic/water/ ammonia, 40:10:49:1, v/v). After development, the ›apergrams were sprayed with 50% ethanol containing 1% Na_2CO_3. The minosugars were visualized under UV light. The spots were eluted with 50% ethanol at 50°C and assayed for sugar concentration (*18*).

Binding of Chitinase to Regenerated Chitin

The affinity adsorption of purified chitinase on regenerated chitin were done by the method of Yabuki et al. (*19*) and Watanabe et al. (*20*).

Protein Determination

This was achieved by the method of Lowry et al. (*21*).

Determination of Total Sugars

Sugar content of the enzymes was determined by the method of Dubois et al. (*22*) as glucose.

RESULTS AND DISCUSSION

Purification of *Aspergillus carneus* Chitinase

The culture broth (3400 mL) was concentrated by ultrafiltration and fractionated by 80% saturation of ammonium sulfate. The resulting precipitate was dissolved in acetate buffer (0.05*M*, pH 5.0) and dialyzed against the same buffer. The enzyme sample was applied to Sephadex G-100 column (2.2×85 cm), equilibrated, and eluted with the same buffer. Protein fractions showed chitinolytic activity (fractions 42-62), Figure 1 were collected and precipitated with 80% ammonium sulfate saturation. The precipitate obtained was dissolved and dialyzed against Tris-HCl buffer (0.02*M*, pH 5.0) and applied to preparative HPLC (protein column, LKB Glaspak TSK G 2000 SW, 30×600 mm). The fractions containing chitinase activity were collected, pooled, and concentrated. Finally the

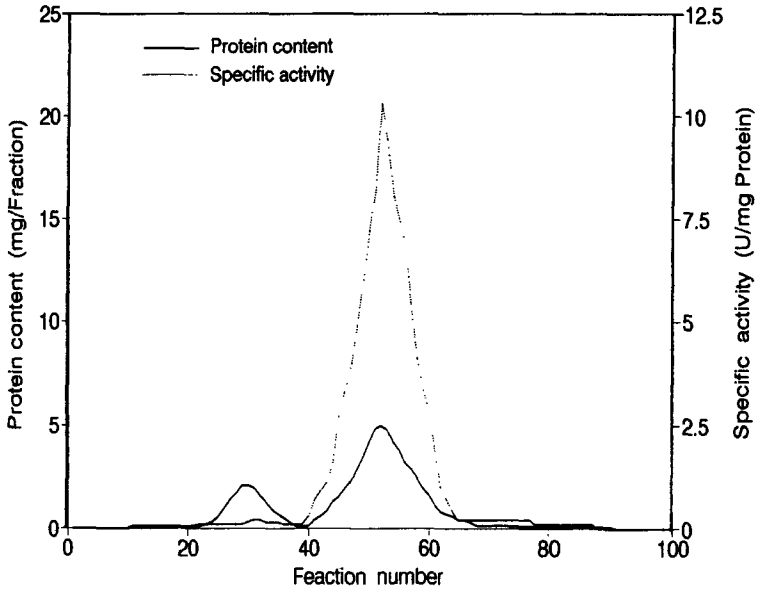

Fig. 1. Gel filtration of *A. carneus* chitinase on Sephadex G-100. The eluates of the fractions 42–62 were collected and used for further purification.

Table 1
Purification Steps of *Aspergillus carneus* Chitinase

Steps	Total protein, mg	Total units, U	Specific activity, U/mg protein	Yield, %	Fold purification
Culture filtrate	3400.0	680	0.20	100.0	1.00
Ultrafiltrate	1686.0	624	0.37	91.76	1.85
Ammonium sulphate saturation (80%)	450.0	500	1.11	73.53	5.55
Gel filteration (Sephadex G-100)	184.0	325	1.77	47.80	8.85
Ammonium sulphate saturation (80%)	98.6	221	2.24	32.50	11.20
Preparative H.P.L.C.	46.0	183	3.97	26.91	19.85
Preparative polyacrylamide gel-electrophoresis	18.0	117	6.5	17.20	32.50

All purification steps were performed at 5°C.

enzyme sample was applied to preparative polyacrylamide gel electrophoresis (PAGE) on several runs. The active fractions were extracted with 0.1*M* acetate buffer, pH 5.0. The yield and purity of chitinase at each purification step are summarized in Table 1. The recovery from the previous steps were 17.2% of the total activity corresponding to 32.5-fold purification.

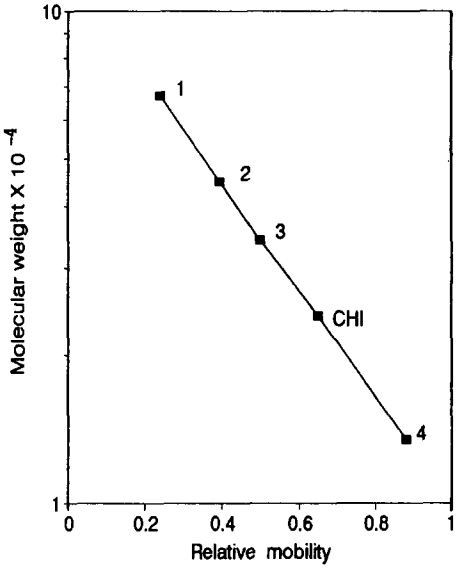

Fig. 2. Determination of the molecular weight of the enzyme (CHI) by SDS-PAGE. The standards were, (1) bovine serum albumin (M.W, 67000), (2) ovalbumin (M.W, 47000), (3) pepsin (M.W, 35000), and (4) lysozyme (M.W, 14000).

Properties of *A. carneus* Chitinase

Ultraviolet Absorption Spectrum

The purified protein in 0.1*M* acetate buffer, pH 5.0 showed a typical absorption spectrum of protein with maximum at 275 nm. The extinction coefficient at 275 nm ($E^{275}_{1\%}$) was calculated to be 12.3.

Molecular Weight of the Enzyme

The molecular weight of *A. carneus* chitinase was estimated by SDS polyacrylamide gel electrophoresis. The results are shown in Fig. 2, and from its mobility, the mol wt was estimated to be about 25000. The molecular weight of *A. carneus* chitinase, was favorably comparable with the values of (25000) *Streptomyces orientalis* (*23*) and (19000) *Streptomyces cinereoruber* (*11*), but smaller than those of reported by Ohtakara et al. (*7*) for *Vibrio* sp. (63000), and by Pegg and Young (*10*) for *Verticillium alboatrum* (63000). However Udea and Arai (*24*) reported a molecular weight of *Aeromonas* sp. chitinase of 111000. This may reflect the variation of mol wt of chitinases from different strains.

Carbohydrate Content

The enzyme was precipitated with 5% trichloroacetic acid (TCA) and the precipitate was found to contain 9.37% (w/w) of carbohydrate, as glucose. Pretreatment of the enzyme solution with 0.1% sodium dodecylsulphate (SDS) and 0.1% urea at 60°C for 2 h, followed by precipitation

Table 2
Amino Acid Composition of Pure *Aspergillus carneus* Chitinase

Amino acid	μmol/mg protein	Mol amino acid/mol enzyme
Aspartic acid + asparagine	0.71	17.75
Threonine	0.81	21.25
Serine	0.67	16.57
Glutamic acid + glutamine	0.57	14.27
Glycine	0.40	10.00
Alanine	0.41	10.25
Valine	0.30	7.50
Cystine	0.02	0.50
Methionine	0.12	3.00
Isoleucine	0.17	4.25
Leucine	0.34	8.50
Tyrosine	0.24	6.00
Phenylalanine	0.20	5.00
Lysine	0.09	2.25
Histidine	0.07	1.75
Arginine	0.13	3.25
Proline	0.20	5.00
Tryptophan	nd*	nd

*nd: not determined.

with 5% TCA also showed the same value of carbohydrate content. This may prove that the carbohydrate residue was not physically attached to the protenic protein of the enzyme. The same findings were reported by Correa et al. (*8*) for *Saccharomyces cerevisiae* chitinase.

Amino Acid Composition

The purified enzyme was analyzed for amino acid contents. The pattern of amino acid composition (Table 2) showed high content of aspartic, threonine, serine and glycine. These amino acids were predominant in the protenic protein of other chitinases from bean leaves (*5*), and *Aeromonas* sp. (*24*).

Effect of pH

The optimum pH of purified enzyme was determined in acetate buffer (0.05*M*) under the standard assay conditions at 45°C. The enzyme was optimally active at pH 5.2 (Fig. 3a). These are similar to the optimal pH of *Trichoderma harzianum* chitinase (*25*).

pH Stability

The pH stability of the enzyme was examined after perincubating the enzyme solution at 30°C for 60 min in different pH(s), followed by adjusting the pH to the value of standard assay system. Then the residual

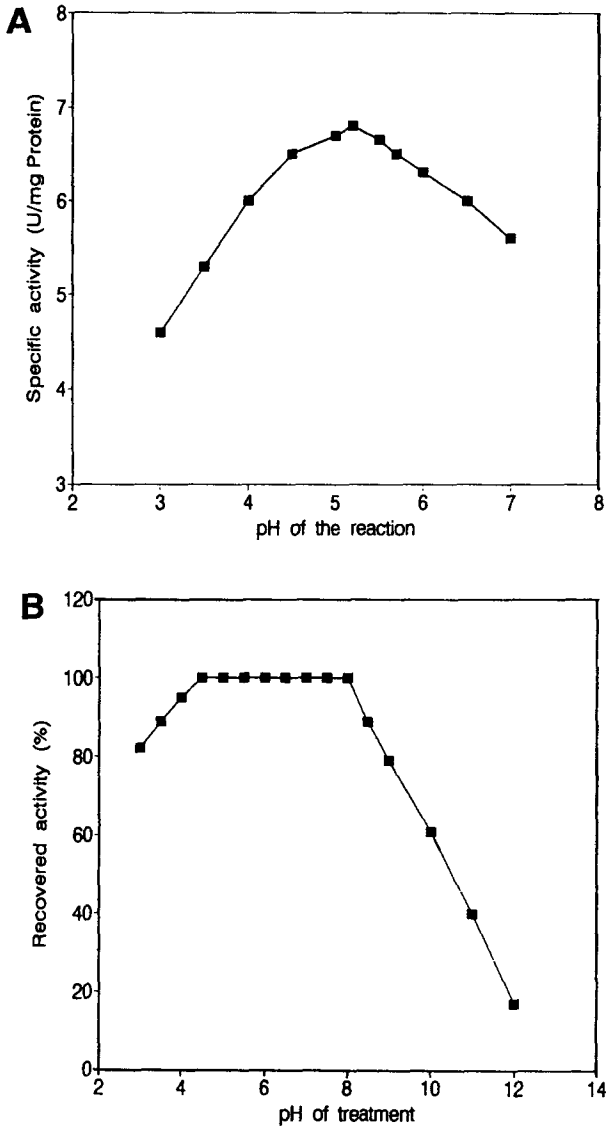

Fig. 3. Effect of pH on the activity and stability of pure *A. carneus* chitinase. Effect of pH on the enzyme activity (a), and effect of pH on the enzyme stability (b).

enzyme activity was assayed under standard conditions. Acetate buffer (0.05*M*, pH 3.7–6.0), Tris malate (0.05*M*, pH 6.5–9), and glycine-NaOH (0.05*M*, pH 10–12) were for enzyme pretreatment. The results (Fig. 3b) showed that the enzyme was stable at pH 3–9. The enzyme compete favorably with *Streptomyces cinereoruber* chitinase (*11*), and more stable than *Aeromonas* sp. chitinase II (*24*).

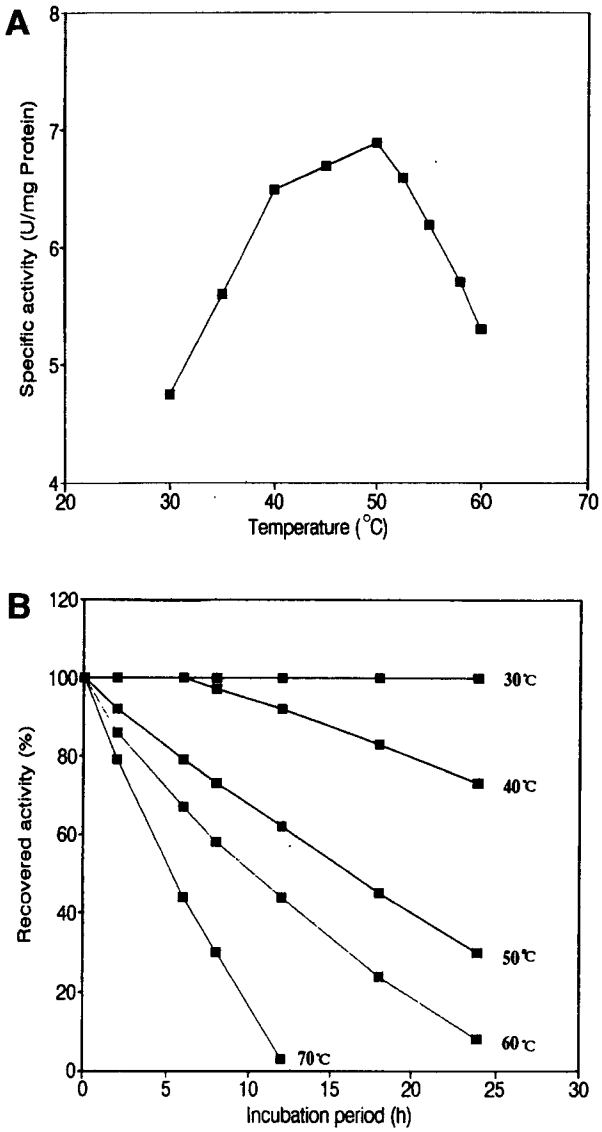

Fig. 4. Effect of temperature on the activity and stability of pure *A. carneus* chitinase. Dependence of the enzyme activity on the temperature (a), and its thermal stability (b).

Effect of Temperature

The optimum temperature was investigated at pH 5.2. As shown in Fig. 4a, the enzyme was most active at 50°C. Temperature optimum of 50°C was reported for *Verticillium albo-atrum* (*10*) and *Streptomyces erythraeus* (*26*).

Thermal Stability

Thermal stability of the enzyme was examined after preincubating of the enzyme solution in acetate buffer (0.05*M*, pH 5.2) at different time intervals. The results (Fig. 4b) showed that *A. carneus* was stable at 30–50°C but was completely inactive at 70°C after heating for 6 h. The enzyme seems to be more stable than *Aeromonas* sp. chitinase (*24*).

K_m and V_{max}

The kinetic constants for chitin hydrolysis by the purified enzyme was also investigated. The low K_m value of *A. carneus* chitinase (4.37 m*M* *N*-acetylglucosamine) may reflect the high affinity of the enzyme to the chitin. This value was favorably comparable to that reported by Robert and Cabib (*9*) for *Serratia marcescens* chitinase (4.4 m*M* *N*-acetylglucosamine). In addition the K_m value of *A. carneus* chitinase was lower than those reported by Reisert (*27*) for *Chytriamyces hyolinus* chitinase (5.7 m*M* *N*-acetylglucosamine). The V_{max} value of *A. carneus* chitinase was 6.22 U/mg protein.

Effect of Metal Ions and Some Reagents

The effect of some metal ions and some reagents on the activity of *A. carneus* chitinase are summarized in Table 3. Of the metal ions investigated, Mg^{2+}, Cu^{2+}, and Ca^{2+} was slightly activated the enzyme. However, Hg^{2+}, I_2, and *p*-chloromercuribenzoate completely inhibited the enzyme activity. The examination of inhibitory effects suggested that sulfhydryl residues participate in the expression of their activities. In general, *A. carneus* chitinase reacts to these reagents in a manner very similar to other chitinases from *Streptomyces orientalis* (*23*), *Vibrio* sp. (*7*), *Verticillium albo-atrum* (*10*), and *Streptomyces cinereoruber* (*11*). However the inhibition pattern of metal ions and organic compounds clearly differed from that reported for *Aeromonas* sp. chitinase (*24*).

Activity Toward Various Substrates

A series of substrates were incubated separately with purified enzyme solution (Table 4). The enzyme had no action on starch, dextran, xylan, and inulin but was active toward chitin and its related compounds. The enzyme attacked chitosan, cellulose, and CMC very slowly. The high specificity of *A. carneus* chitinase justifies its suitability for chitin hydrolysis.

Binding of Chitinase to Regenerated Chitin

The enzyme was strongly adsorbed to regenerated chitin and cellophane tubes. In addition during the saccharification of chitin utilizing *A. carneus* chitinase (data not shown), the enzyme tightly adsorbed to crabshell chitin and released only after the hydrolysis of more than 90% of the substrate. Similar findings were reported for *Aeromonas hydrophila* chitinase (*19*).

Table 3
Effect of Various Chemicals on the Activity of Pure *Aspergillus carneus* Chitinase

Reagent	Relative activity (%)	
	5 m*M*	10 m*M*
None	100.00	100.00
NaCl	97.30	91.20
$CaCl_2$	102.30	107.40
$AgNO_3$	92.43	87.55
$CoCl_2$	84.30	77.30
$MgCl_2$	109.30	114.30
$CuCl_2$	104.20	111.30
$FeSO_4$	91.30	80.40
$HgCl_2$	41.30	30.00
$ZnCl_2$	97.30	94.30
I_2	00.00	00.00
PCMB*	07.30	00.00
SDS**	97.30	93.50
EDTA***	97.30	95.44
MIA****	95.44	91.67
Cystein	101.45	101.88

*p-Chloromercuribenzoate.
**Sodium dodecylsulphate.
***Ethylenediaminetetraacetate (sodium salt).
***Monoiodoacetic acid.

Table 4
Substrate Specificity of *Aspergillus carneus* Chitinase

Substrate	Substrate concentration, mg/mL	Specific activity, U/mg protein
Shrimp chitin	10.0	0.49
Colloidal chitin	10.0	7.42
Glycol chitin	10.0	8.11
Chitobiose	5.0	0.12
Chitotriose	5.0	0.94
Chitotetraose	5.0	3.22
Chitopentaose	5.0	6.37
Chitohexaose	5.0	8.68
Chitosane	5.0	0.04
Cellulose (Avicell)	50.0	0.02
Carboxymethylcellulose	10.0	0.07
Starch	10.0	0.00
Dextrin	10.0	0.00
Dextran	10.0	0.03
Laminaran	5.0	0.79
Xylan	10.0	0.00
Inulin	5.0	0.00

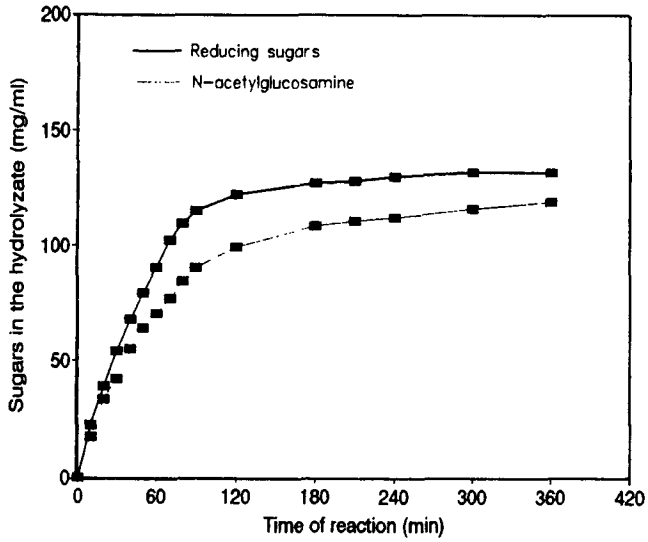

Fig. 5. Hydrolysis course of chitin by the purified *A. carneus* chitinase.

Hydrolysis of Chitin by *A. carneus* Chitinase

The hydrolyzate products of chitin (colloidal chitin) by the action of purified chitinase was investigated during the course of hydrolysis. The results (Fig. 5) showed that *N*-acetylglucosamine was the major ingredient with very little amounts of oligomers. The distribution of the hydrolyzate products after 60 min (Fig. 6) showed that *N*-acetylglucosamine was the major amino sugar in the hydrolyzate (about 90%). The early formation of *N*-acetylglucosamine with very little association of other oligomers and chitobiose, gives evidence for this enzyme to be exochitinase.

The Lytic Action of *A. carneus* Chitinase

The lytic action of *A. carneus* chitinase on *Aspergillus niger* and *Micrococcus lysodeikticus* cell walls was investigated. The enzyme did not lyse completely the mycellium of *A. niger* cell wall. However the combination of *A. carneus* chitinase and exogenous α-1,3-glucanase degraded with mycelium markedly and release the protoplast from a 3-d-old culture of *A. niger*. This coincides with the observation of de Vries and Wessels (*28*) on the release of protoplast from *Schizophyllum comune*. Since the cell wall of both *Schizophyllum commune* and *A. niger* are composed mainly of α-glucans and chitin (*29*).

Interestingly, *A. carneus* chitinase was able to hydrolyze the *Micrococcus lysodeikticus* cell wall. However, the protoplast was released after 48h. Further studies on the lytic action of *A. carneus* chitinase is now under investigation, and will be described elsewhere.

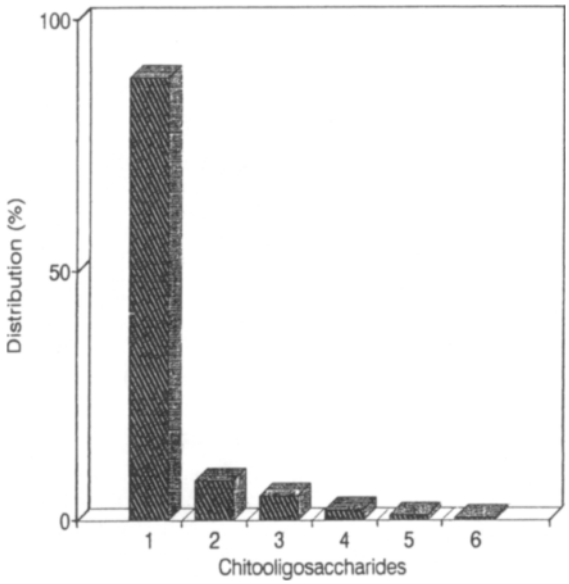

Fig. 6. Distribution of hydrolyzate products of chitin by the action of pure *A. carneus* chitinase. The authantic: 1, *N*-acetylglucosamine, 2, chitobiose, 3, chitotriose, 4, chitotetraose, 5, chitopentaose, and 6, chitohexaose.

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REFERENCES

1. Muzzarelli, R., Jeuniaux, C., and Gooday, G. W. (1986), in *Chitin in Nature and Technology,* Pergamon Press, New York, p. 435.
2. Wood, W. A. and Kellogg, S. T. (1988), in *Methods in Enzymology,* vol. 161, Colowick, S. P., and Kaplan, N. O., eds., Academic, New York, p. 462.
3. Sakai, K., Nanjo, F., and Usui, T. (1990), *Denpun Kagaku.* **37,** 79.
4. Ohtakara A., Ogata, H, Taketomi, Y., and Mitsutomi, M. (1984), in *Chitin, Chitosan and Related Enzymes,* Zikakis, J. P., ed., Academic, New York, p. 147.
5. Abeles, F. B., Basshart, R. P., Forrence, L. E., and Habing, W. H. (1971), *Plant Physiol.* **47,** 129.
6. Deshpande, M. V. (1986), *J. Sci. Ind. Res.* **45,** 273.
7. Ohtakara, A., Mitsutomi, M., and Uchida, Y. (1979), *J. Ferment. Technol.* **57,** 169.
8. Correa, J. U., Elango, N., Polackeck, I., and Cabib, E. (1982), *J. Biol. Chem.* **257,** 1392.

9. Roberts, R. L. and Cabib, E. (1982), *Anal. Biochem.* **127,** 402.
10. Pegg, G. H. and Young, D. H. (1982), *Physiol. Plant. Pathol.* **21,** 389.
11. Okazaki, K. and Tagawa, K. (1991), *J. Fermnt. Bioeng.* **71,** 237.
12. Sherief, A. A., El-Sawah, M. M. A., and Abdel-Naby, M. A. (1991), *Appl. Microbiol. Biotechnol.* **35,** 228.
13. Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem.* **217,** 959.
14. Laemmli, U. K. (1970), *Natur (London),* **227,** 680.
15. Weber, K. and Osborn, M. (1969), *J. Biol. Chem.* **244,** 4406.
16. Rondle, C. J. and Morgan, W. T. (1955), *Biochem. J.* **61,** 586.
17. Somogyi, M. (1952), *J. Biol. Chem.* **195,** 19.
18. Charlemagne, D. and Jolles, P. (1967), *Soc. Chim. Biol.* **49,** 1103.
19. Yabuki, M., Mizushina, K., Amatatsu, T., Ando, A., Fujii, T., Shimada, M., and Yamashita, M. (1986), *J. Gen. Appl. Microbiol.* **32,** 25.
20. Watanabe, T., Oyanagi, W., Suzuki, K., and Tanaka, H. (1990), *J. Bacteriol.* **172,** 4017.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265.
22. Dubois, M., Gilles, A., Hamilton, J., Rebers, P., and Smith, F. C. (1956), *Anal. Chem.* **28,** 350.
23. Tominaga, Y. and Tsujisaka, Y. (1976), *Agric. Biol. Chem.* **40,** 2325.
24. Udea, M. and Arai, M. (1992), *Biosci. Biotech. Biochem.* **56,** 460.
25. Eld, Y., Chet, I., and Henis, Y. (1982), *Can. J. Microbiol.* **28,** 719.
26. Hara, S., Yamamura, Y., Fujii, Y., Mega, T., and Ikenaka, T. (1989), *J. Biochem.* **105,** 484.
27. Reisert, P. S. (1972), *Mycologia.* **64,** 288.
28. de Vries, O. M. H. and Wessels, J. G. H. (1973), *J. Gen. Microbiol.* **76,** 319.
29. Siehr, D. H. (1976), *Can. J. Biochem.* **54,** 130.